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Expressed sequence tag analysis of the response of apple (*Malus* x *domestica* 'Royal Gala') to low temperature and water deficit

Michael Wisniewski[a,*], Carole Bassett[a,*], John Norelli[a], Dumitru Macarisin[a], Timothy Artlip[a], Ksenija Gasic[b] and Schuyler Korban[b]

[a]United States Department of Agriculture – Agricultural Research Service (USDA-ARS), Appalachian Fruit Research Station, 2217 Wiltshire Road, Kearneysville, WV 25430, USA
[b]Department of Natural Resources and Environmental Sciences, University of Illinois, 310 ERML, 1201 W. Gregory Drive, Urbana, IL 61801, USA

Correspondence
*Corresponding author,
e-mail: michael.wisniewski@ars.usda.gov; carole.bassett@ars.usda.gov

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Leaf, bark, xylem and root tissues were used to make nine cDNA libraries from non-stressed (control) 'Royal Gala' apple trees, and from 'Royal Gala' trees exposed to either low temperature (5°C for 24 h) or water deficit (45% of saturated pot mass for 2 weeks). Over 22 600 clones from the nine libraries were subjected to 5′ single-pass sequencing, clustered and annotated using BLASTX. The number of clusters in the libraries ranged from 170 to 1430. Regarding annotation of the sequences, BLASTX analysis indicated that within the libraries 65–72% of the clones had a high similarity to known function genes, 6–15% had no functional assignment and 15–26% were completely novel. The expressed sequence tags were combined into three classes (control, low-temperature and water deficit) and the annotated genes in each class were placed into 1 of 10 different functional categories. The percentage of genes falling into each category was then calculated. This analysis indicated a distinct downregulation of genes involved in general metabolism and photosynthesis, while a significant increase in defense/stress-related genes, protein metabolism and energy was observed. In particular, there was a three-fold increase in the number of stress genes observed in the water deficit libraries indicating a major shift in gene expression in response to a chronic stress. The number of stress genes in response to low temperature, although elevated, was much less than the water deficit libraries perhaps reflecting the shorter (24 h) exposure to stress. Genes with greater than five clones in any specific library were identified and, based on the number of clones obtained, the fold increase or decrease in expression in the libraries was calculated and verified by semiquantitative polymerase chain reaction. Genes, of particular note, that code for the following proteins were overexpressed in the low-temperature libraries: dehydrin and metallothionein-like proteins, ubiquitin proteins, a dormancy-associated protein, a plasma membrane intrinsic protein and an RNA-binding protein. Genes that were upregulated in the water deficit libraries fell mainly into the functional categories of stress (heat shock proteins, dehydrins) and

Abbreviations – ELIPs, early light-inducible proteins; ICE1, inducer of CBF expression 1; LEA, late-embryogenesis abundant; LT, low temperature stressed; Mdlv, non-stressed leaf tissue; Mdst, non-stressed stem tissue (served as a control for both xylem and bark tissues); Mdrt, non-stressed root tissue; PPFD, photosynthetic photon flux density; WD, water deficit stressed.

photosynthesis. With few exceptions, the overall differences in downregulated genes were nominal compared with differences in upregulated genes. The results of this apple study are similar to other global studies of plant response to stress but offer a more detailed analysis of specific tissue response (bark vs xylem vs leaf vs root) and a comparison between an acute stress (24-h exposure to low temperature) and a chronic stress (2 weeks of water deficit).

Introduction

Low temperature and drought are two common abiotic stresses to which plants are subjected. Together, temperature and water availability are the primary determinants of the global distribution of major vegetation biomes (Graham et al. 2006, Street et al. 2006). Woody plants have evolved mechanisms of resistance and adaptation to both low temperature and water limitation. For example, cold hardiness is a complex trait that includes acclimation, midwinter hardiness and deacclimation, and is influenced by a number of contributing factors (Juntilla et al. 2002, Kalberer et al. 2006, Welling and Palva 2006, Wisniewski and Arora 2000a, 2000b, Wisniewski et al. 1993, 2003). Likewise, drought adaptation is controlled by complicated interactions between plant anatomy, physiology and biochemistry, all of which are directly or indirectly under genetic control (Arora et al. 2000, Bohnert et al. 1995, Lim et al. 1998, Street et al. 2006; Verslues et al. 2006, Welling and Palva 2006).

Cold acclimation involves the activation of multiple mechanisms that collectively contribute to augment freezing tolerance. Such changes include the accumulation of cryoprotective molecules (sugars, compatible solutes and proteins), and also alterations in membrane composition (Fowler and Thomashow 2002, Guy 2003, Zhang et al. 2004). In the case of *Arabidopsis*, global changes in gene expression in response to low temperature have been extensively documented (Fowler and Thomashow 2002, Shinozaki et al. 2003, Zarka et al. 2003). In particular, a large family of cold-regulated genes has been identified as well as a family of transcription factors (CBF1-3) that regulates subsets of cold-responsive genes by binding to a C-repeat element in the promoter region of target genes (Jaglo-Ottosen et al. 1998, 2001). Recently, another transcription factor, inducer of CBF expression 1 (ICE1), was identified and its cold-responsive transcriptome elucidated (Chinnusamy et al. 2003, Lee et al. 2005). Among the genes regulated by the ICE/CBF pathway are those encoding pathogenesis-related proteins like β-1,3-glucanases, protective proteins such as defensin, and proteins with various functions, including dehydrins and early light-inducible proteins (ELIPs).

Expressed sequence tag (EST) analyses of woody plants in response to low temperature have been conducted in blueberry (*Vaccinium* spp.) (Dhanaraj et al. 2004, 2007), *Rhododendron* (Wei et al. 2005), peach (*Prunus persica*) (Bassett et al. 2006) and *Populus* leaves (Nanjo et al. 2004). Other EST studies on woody plants include analyses of aroma production (Schaffer et al. 2007), fruit development (Park et al. 2006) and a general EST overview (Newcomb et al. 2006) in apple, grape (*Vitis vinifera*) ESTs in leaf and berry (Ablett et al. 2000), Norway spruce (*Picea abies*) ESTs during budburst (Yakovlev et al. 2006), and Maritime pine (*Pinus maritima*) ESTs in response to water deficit (Dubos and Plomion 2003). Thus far, studies of photoperiod and low-temperature-induced gene expression in woody plants indicate that a major family of genes that appear to be consistently upregulated is the late-embryogenesis-abundant (LEA) gene/protein family. In particular, the dehydrin subfamily of LEA proteins (group II) has been extensively characterized in woody plants (Arora and Wisniewski 1994, Karlson et al. 2003, 2004, Lim et al. 1999, Welling et al. 2004, Wisniewski et al. 2004, 2006). The observation that dehydrins are responsive to low temperature is not surprising in view of their reported cryoprotective and antifreeze properties (Wisniewski and Arora 2000b).

Drought tolerance involves adaptation through a variety of morphological, physiological and biochemical changes (Baker 1982, Bengtson et al. 1978, Ingram and Bartels 1996). Stomatal closure, particularly during elevated temperatures, is a common mechanism for preventing daily water loss, and the size and density of stomates correlates with water use efficiency (Dudley 1996). Other parameters, such as root depth, leaf size and trichome density have also been linked to water use efficiency (Ingram and Bartels 1996).

Global analysis of gene expression associated with water deficit treatment has been reported in a number of herbaceous and woody species (Bassani et al. 2004, Buitink et al. 2006, Jia et al. 2006, Oono et al. 2003, Plomion et al. 2006, Pratt et al. 2005, Seki et al. 2002, Wong et al. 2006). Comparison of genes responding to dehydration and other stresses, such as cold and salt treatment indicates that there is some overlap among stress-responsive genes (Bray 2004, Sreenivasulu et al. 2007), although the degree of overlap varies depending on the plant. For example, Rabanni et al. (2003) and Seki

et al. (2002) found 14 and 11% overlap in cold-, drought- and salinity-responsive genes in rice and *Arabidopsis*, respectively. In contrast, Wong et al. (2006) only found 4% overlap among genes responding to the same three stresses in *Thellungiella*. Although overlapping responses are often seen at the level of gene family, there is substantial evidence that individual family members respond differently to various stresses. For example, Nylander et al. (2001) observed that some dehydrins responded to either cold or drought, while others responded to both. This is not unexpected in view of the fact that CBF/DREB1 and DREB2 transcription activation factors bind to the same core element in the promoters of genes regulated by cold or drought, respectively (Gilmour et al. 1998, Liu et al. 1998).

Little information exists on how temporal patterns of gene expression differ between *Arabidopsis* and woody plants during cold acclimation and exposure to dehydration or how their transcriptome profiles change over time during cold acclimation (Bassett et al. 2006, Renaut et al. 2004). Additionally, no information exists on how tissues within the same plant that exhibit contrasting mechanisms of cold hardiness (e.g. bark which exhibits equilibrium freezing vs xylem which exhibits deep supercooling) differ in their patterns of transcriptome or proteome expression profiles. ESTs, are an efficient and rapid means to identify genes affected by environmental stresses, especially when comprehensive oligo arrays are not readily available. Recently, large-scale EST analyses of apple (*Malus* × *domestica)* were reported by Newcomb et al. (2006) and Park et al. (2006). However, neither study examined ESTs expressed during exposure to cold or drought conditions. In the current study we present an EST analysis of the response of different apple tissues to either low temperature or water deficit. Over 22 600 single-pass 5′-end nucleotide sequences were generated from stressed and non-stressed apple trees (cv. Royal Gala) representing nine different libraries. The generation of these ESTs was part of a large project to enhance the number of apple ESTs available in public databases.

Materials and methods

Plant material

Apple seedlings (*Malus* × *domestica* cv. 'Royal Gala') were initially propagated in tissue culture at the USDA-ARS-NAA-AFRS facility (Kearneysville, WV) essentially as per Norelli et al. (1988) and Ko et al. (2002), with root induction as per Bolar et al. (1998). After subsequent rooting and potting steps, they were transferred to a glasshouse where they were grown under supplemental lighting to maintain the day length at 16 h, and a temperature range of 20–35°C. Trees were watered daily, and refertilized after 5 months. Trees were in the glasshouse for a total of 6 months, with stem calipers ranging from 0.5 cm to slightly more than 1.0 cm, and heights varying from 1 to 2 m.

Photoperiod and temperature treatments

Seventy trees were placed in a Conviron PGV36 growth chamber at 25°C with a continuous 24-h light cycle, photosynthetic photon flux density (PPFD) was approximately 500 μmol photons m^{-2} s^{-1}. The trees were watered daily and were subjected to these conditions for 1 week. Four time zero (t_0) trees were destructively harvested immediately prior to the low temperature treatment. Bark and leaf samples were immediately frozen in liquid N_2 and stored at −80°C. The chamber was then reset to 5°C, but continued on the 24-h light cycle. Upon the chamber reaching 5°C, trees were harvested in groups of four, as above, at the following time points: 15and 30 min, 1, 2, 4, 8, 12, 24, 48 and 96 h, and 1 and 3 weeks. Only the 24-h samples were used in the current study.

Water deficit

Twenty five trees were placed in a Conviron PGV36 growth chamber (Conviron) at 25°C day (16 h)/18°C night (8 h) with light at 500 μmol photons m^{-2} s^{-1} PPFD. Water deficit was imposed essentially as described by Artlip and Wisniewski (1997). Briefly, water was withheld from 10 trees until the pot masses were at 45% of the saturated mass and maintained at this level for 2 weeks by adding back water to the 45% level, while 10 control trees were maintained at the saturated mass by daily watering. After 2 weeks of water limitation, five water deficit trees were destructively harvested by sampling roots, bark and leaves that were immediately frozen in liquid N_2 and stored at −80°C. The harvested leaves were typically taken from leaf positions 7 through 12 (measured from the youngest visible leaf at the apex), and typically measured from 6.5 to 9.0 cm in length. These leaves are considered to be at or near full expansion.

Construction of cDNA libraries

Primary cDNA libraries were prepared from leaves, bark, xylem and roots of young apple trees exposed to either 5°C or water deficit conditions. Total RNA was extracted from frozen tissues using a modified Cetyltrimethylammonium bromide (CTAB) method (Gasic et al. 2004). Poly(A)$^+$mRNA was isolated twice from total RNA from each sample using the Oligotex Direct mRNA kit (Qiagen, Valencia, CA). mRNA was reverse transcribed

into double-stranded cDNA using a modified oligo$_{18}$(dT) primer with an identifying tag sequence. cDNAs from replicate extractions were pooled in equal amounts before adaptor ligation. cDNA libraries were constructed following procedures described in Bonaldo et al. (1996) and Soares et al. (1994). Double-stranded cDNAs were size-selected to enrich for molecules >500 bp, adaptored with *Eco*RI adapters (Promega, Madison, WI) at both ends, and then digested with *Not*I. cDNAs were then directionally cloned into *Eco*R1 (5′) – *Not*I (3′) digested pBluescript II SK(+) phagemid vector (Stratagene, La Jolla, CA). Ligated cDNA fragments were transformed into *Escherichia coli* ElectroMax DH10B host cells.

Clone preparation and sequencing

To confirm presence and size of the inserts, 192 white colonies per library were randomly handpicked and grown overnight in 200 μl of YT medium supplemented with ampicillin and glycerol in two 96-well plates. Polymerase chain reaction (PCR) reaction was performed using primers M13 universal forward and reverse to amplify cloned inserts. Agarose gel (1%) analysis of PCR products revealed the presence and average size of the cloned fragments (465 bp). Grown plates were forwarded to the W.M. Keck Sequencing Center (University of Illinois Champaign-Urbana, IL) to verify quality of cloned fragments by sequencing. cDNA libraries were then plated, and individual colonies were robotically picked and assigned a unique identifier. Glycerol stocks of cDNA clones were sent to the Washington University Genome Sequencing Center (Washington University, St Louis, MO) for high-throughput 5′ single-pass sequencing. Clones were transferred into 96-well blocks and incubated at 37°C for 24 h at 297 rpm in an incubator shaker. Clones were processed according to Marra et al. (1999) using a high-throughput 96-well microwave protocol. Dideoxy terminator sequencing reactions were conducted as described in Hillier et al. (1996). A total of nine libraries were sequenced as follows: Mdlv, non-stressed leaf tissue; Mdst, non-stressed stem tissue (served as a control for both xylem and bark tissues); Mdrt, non-stressed root tissue; Mdltl, leaf tissue exposed to 5°C for 24 h; Mdltb, bark tissue exposed to 5°C for 24 h; Mdltx, xylem tissue exposed to 5°C for 24 h; Mdwdl, leaf tissues exposed to water deficit for 2 weeks; Mdwdb, bark tissue exposed to water deficit for 2 weeks, and Mdwdr, root tissue exposed to water deficit for 2 weeks.

Editing, clustering, and annotation of the ESTs

EST's were vector trimmed using the software CodonCode Aligner version 1.2.0 (CodonCode Corporation, Dedham, MA). Each of the nine data sets was clustered individually using either CodonCode Aligner (PHRAP) and/or Seqtools 8.3 (http://www.seqtools.dk) assembly programs using standard assembly parameters: (minimum % identity: 70, minimum overlap: 25 bp, minimum score: 20, maximum unaligned overlap: 50, matchscore: 1, mismatch penalty: −2, gap penalty: −2). A list of contigs and singletons was generated for each individual library. Each contig or singleton was assumed to represent a unique transcript. Unique transcripts were annotated by blastx against NCBI's Plant_Ref_seq and Arab._ref_seq databases as a reference set in Pymood (Allometra, Davis, CA) or using a web-based program blast2go (http://www.blast2go.de).

Quantitative assessment of ESTs in different cDNA libraries

Clustered sequences were identified and those containing five or more individual sequences were chosen for quantification. The cutoff value of a minimum of five sequences was based on a 95% confidence interval calculated for 1000 randomly selected clones from a cDNA library containing 0 representatives of a given sequence (Audic and Claverie 2006). The number of sequences within a given cluster was divided by the total number of sequences in that library and multiplied by 100 to yield the percentage of each gene found in the library. To determine the fold difference for upregulated sequences, the percentage of each gene found in the treated library was divided by the percentage of that gene found in the appropriate control library. For downregulated sequences, the fold difference was calculated by dividing the percentage of the given gene in the control by the percentage of that gene in the appropriate treated library. If no sequences for a given gene were found in a library under analysis (observed sequence = 0), a range was computed based on the possibility that there could have been from 1 to 4 actual copies of that gene even though zero sequences were observed. Inclusion of a 'greater than' or 'less than' sign indicates the possibility that the actual number of sequences could have actually been zero, which could not be used to compute a real value.

Confirmation of gene expression by semiquantitative RT-PCR

Primer sequences for genes of interest are listed in Table 1. RNAs for reverse transcription were isolated using the Plant RNA Extraction Reagent (Invitrogen, Carlsbad, CA) and analyzed on a formaldehyde gel to determine quality. First-strand cDNAs were transcribed using the Advantage RT-for-PCR kit (Clontech, Mountain

Table 1. List of primers and predicted product sizes for RT-PCR confirmation analysis. The PCR cycling conditions are described in Materials and methods. [a]Gene abbreviations are as follows: MdDhn1, Y_nK_3 dehydrin; MdHSP18, 18.1 kDa heat shock protein; MdERC, ethylene-responsive transcriptional coactivator; MdPepPro, peptidylprolyl-*cis/trans*-isomerase; MdDhn3. SK_3 dehydrin; MdLHCB2.2, light harvesting complex B type II Chl *a/b*-binding protein; MdMTHFR, methylenetetrahydrofolate reductase 2; MdGerGer, geranylgeranyl reductase; Sub7, Subunit7 of the peach 26S proteasome. [b]Product size in base pairs predicted from sequence information.

Gene[a]	Forward primer	Forward primer sequence	Reverse primer	Reverse primer sequence	Size[b]
MdDhn1	Xero2for(2361)	5′-ctggtggttggacgtagggatgac-3′	Xero2rev(2740)	5′-cactcgcgcacgtaagaaagaaat-3′	648
MdHSP18	18.1kDaHSP(136)	5′-cccggcaaaaacccagcatcta-3′	18.1kDaHSP(560)	5′-cagttcagcaccccgttctccatc-3′	434
MdERC	ERCfor(387)	5′-tcggatttgcgggatttgttgtaa-3′	ERCrev(617)	5′-caatggcccttctacttcctgtgc-3′	232
MdPepPro	Peptidylfor(219)	5′-ctccggcaagcccctccactacaa-3′	Peptidylrev(534)	5′-ccccaccttctcgatgttcttcac-3′	315
MdDhn3	Cor47for(178)	5′-tcgccgttgctgtcgctcttt-3′	Cor47rev(1118)	5′-catcttcctcctcatcacttg-3′	940
MdLHCB2.2	LHCB2.2F248	5′-aaccgtgagcttgaggtgat-3′	LHCB2.2R896	5′-cagcggtttcaatacaagca-3′	649
MdMTHFR	MdMTHFRF3	5′-gccgtgtgtcggtgcagact-3′	MdMTHFRR3	5′-aggctccaaggcagcggtaa-3′	801
MdGerGer	MdGerF2	5′-tgttctggtgaaggcatcta-3′	MdGerR2	5′-acaacaattgtgccgtgtgt-3′	399
Sub7	Ch2313F	5′-gatcctccaaagggtctcctc-3′	Pam181R	5′-cggtgttgcactgaacttctgg-3′	657

View, CA). cDNAs were separated on agarose gels, stained with SYBR Gold and quantified via an image analyzer (Typhoon Trio, GE/Amersham Biosciences, Piscataway, NJ). cDNAs within an experimental group were normalized to the least concentrated sample. Multiple dilutions for each cDNA were tested with each primer set to determine the optimum template concentration. Primers for 26S rRNA (kindly provided by Dr Ann M. Callahan, USDA, ARS) and for the 26S proteosome subunit 7 (Bassett et al. 2005) served as internal and loading controls for the upregulated and downregulated series, respectively. For upregulated genes, 'Touchdown' RT-PCR was performed using HotStarTaq plus (Qiagen) as follows: 94°C for 5 min, then 10 cycles of 94°C for 30 s, 70°C annealing for 30 s, 72°C extension for 45 s, decreasing 1°C for each cycle during the annealing phase and followed by 16–22 cycles of 94°C for 30 s, 57–60°C for 30 s, 72°C for 45 s with a final extension of 70°C for 10 min. For downregulated genes, RT-PCR using HotStarTaq consisted of 94°C for 15 min, then 30 (MdLHCB2.2 and MdMTHFR) or 40 (MdGerGer) cycles of 94°C 1 min, 60°C 1 min, 72°C 1–2 min with a final extension at 72°C of 5–10 min.

Results

Clustering and transcript analysis

ESTs from a total of nine libraries were sequenced (Table 2). These libraries were derived from potted, apple (cv. Royal Gala) seedlings exposed to 5°C for 24 h or water deficit for 2 weeks. The low temperature libraries were made from leaf (MdLTL), xylem (MdLTX) or bark (MdLTB) tissues, whereas the water deficit libraries were obtained from leaf (MdWDL), bark (MdWDB) and root (MdWDR) tissues. Previously sequenced libraries from non-stressed 'Royal Gala' trees were used as comparative controls. These libraries were obtained from leaf (Mdlv), root (Mdrt) and stem (Mdst) tissues. The latter library served as a control for the MdLTX , MdLTB and MdWDB libraries. The number of sequences obtained from each library ranged from a low of 1674 sequences (MdWDL) to

Table 2. Number of ESTs, clustering and annotation of transcripts from nine cDNA libraries as described in the Materials and methods.

	Total number of ESTs	Clustering ESTs		Similar to known function genes, n (%)	Similar to unknown function genes, n (%)	No similarity found (novel), n (%)
		Number of contigs	Number of singeltons			
Mdltl	1762	197	1062	883 (70.1)	192 (15.3)	184 (14.6)
Mdltx	1774	206	1177	973 (70.4)	182 (13.2)	228 (16.5)
Mdltb	1805	183	1212	943 (67.6)	189 (13.5)	263 (18.9)
Mdwdl	1721	170	988	752 (65.2)	173 (15.0)	229 (19.8)
Mdwdb	1674	165	1066	797 (64.7)	177 (14.4)	257 (20.9)
Mdwdr	1791	181	1252	1011 (67.1)	172 (11.4)	324 (21.5)
Mdlv	2857	494	0	336 (68.0)	29 (5.8)	129 (26.2)
Mdst	7160	1430	3912	3539 (66.2)	739 (13.8)	1064 (19.9)
Mdrt	2108	214	1409	1158 (71.3)	209 (12.9)	256 (15.8)

a high of 7160 sequences (Mdst). The total number of sequences used in this study was 22 660. As indicated in Table 2, the number of clusters in the libraries ranged from 170 to 1430. BLASTX analysis of the resulting clusters and singletons indicated that 65–72% had a high similarity (e-value) to genes of known function, 6–15% genes with no functional assignment and 15–26% were completely novel.

To determine the similarity and differences in the EST libraries, a number of sequences were progressively subtracted from each library. This analysis is presented in Table 3, where the per cent similarity and uniqueness of each of the tissue-/stress-specific libraries is shown in relation to other control- or stress-specific libraries, where A = tissue-specific sequences (clusters plus singletons) minus the tissue-specific control sequences; B = tissue-specific sequences minus control sequences from all tissues and C = B minus the treatment-specific sequences from the other two tissue-specific libraries. As an example, for the MdLTL library, A = leaf tissue ESTs after exposure to 5°C for 24 h minus ESTs from untreated Mdlv (control library); B = leaf tissue ESTs after exposure to 5°C for 24 h minus ESTs from all untreated, control libraries (Mdlv, Mdst and Mdrt), and C = B minus ESTs from MdLTX and MdLTB libraries.

After 24-h exposure to low temperature, leaf tissue appeared to be very responsive because 77% of the library appeared to be unique when just tissue-specific control ESTs were subtracted out. This compared to 44 and 48% unique sequences for the xylem and bark libraries. However, the xylem library appeared to contain the highest percentage of unique sequences (42%) compared with 27% for leaf and bark libraries, when sequences common to all the control libraries and other two tissues (bark and leaf) were subtracted out. The water deficit libraries exhibited a high percentage of unique sequences, ranging from 50 to 79% when just tissue-specific control ESTs were subtracted out. The percentage of unique sequences in each of the water deficit, tissue-specific libraries was very similar when all control sequences were subtracted out, as well as sequences common to other tissues, ranging from 32 to 35%. The high level of unique sequences in the water deficit libraries may reflect an overall shift in gene expression after 2 weeks of water deficit. In contrast, the lower number of unique sequences in the low-temperature libraries may reflect the shorter duration of the stress. Annotated sequences and their expression in the different stress libraries is presented in Supplementary material (Table S1).

The data presented in Table 3 are only an estimate of uniqueness and similarity because several factors can contribute to whether or not a gene is cloned and sequenced. These factors include: the quality of the RNA extraction and reverse transcription into cDNA, the copy number of the gene present in the cDNA library and the number of clones sequenced from each library. Presence or absence of a gene in a particular library may be the reflection of random sampling error; however, the percentages of similarity are in line with or actually higher than other reports (Bhalerao et al. 2003, Dhanaraj et al. 2004, Wei et al. 2005). We also combined a number of libraries and subtracted the common sequences from each of the stress-related libraries to compensate for random sampling error. A more quantitative approach to identifying uniquely expressed genes is presented later in the study.

Table 3. Percentage of unique and similar transcripts in cDNA libraries after a series of subtractions. Mdltl, apple leaf tissue exposed to 5°C for 24 h; Mdltx, apple xylem tissue exposed to 5°C for 24 h; Mdltb, apple bark tissue exposed to 5°C for 24 h; Mdwdl, apple leaf tissue subjected to water deficit for 2 weeks; Mdwdb, apple bark tissue subjected to water deficit for 2 weeks; Mdwdr, apple root tissue subjected to water deficit for 2 weeks. A, tissue-specific sequences minus tissue-specific control sequences; B, tissue-specific sequences minus all control sequences; C, B minus treatment-specific sequences from other tissue-specific libraries. U, unique sequences; S, similar sequences.

Library	A U/S	B U/S	C U/S
Mdltl	77/23	33/67	27/73
Mdltx	44/56	44/56	42/58
Mdltb	48/52	35/65	27/73
Mdwdl	79/21	38/62	32/68
Mdwdb	50/50	39/61	35/61
Mdwdr	70/30	38/62	33/67

Putative identification and functional category assignment

To group the ESTs of each library into functional categories, each EST was compared with the non-redundant protein database utilizing either PYMOOD or BLAST2GO, as described in Materials and methods. Prior to conducting this analysis, ESTs were grouped into one of three treatment categories: control (all the control library sequences combined), low temperature (LT; all the low-temperature library sequences combined) or water deficit (WD; all the water deficit library sequences combined). The resulting annotations for each combination were sorted alphabetically. Each identified sequence was then manually assigned to one of 10 functional categories or to either an unclassified or unidentified category (Table 4). With some modification, the functional categories were based on the main groups of a system developed by Ablett et al. (2000) and adapted by Dhanaraj et al. (2004) and

Table 4. Abundance of transcripts, represented as a percentage of all ESTs in each category, expressed as the percentage of ESTs in each functional category for control, low-temperature–stressed (LT) and water-deficit-stressed (WD) libraries. Bold and an up (↑) or down (↓) arrow indicates that there was significant difference (more than 25%) between control and acclimated library in the percentage of cDNAs belonging to a functional category as a whole. Bold and an up (↑↑) or down (↓↓) arrow indicates that there was two-fold or higher difference in the percentage of cDNAs in acclimated library compared with control library.

	Percent				
Functional category	Control	LT		WD	
General metabolism					
Amino acid, nucleotide, nitrogen/sulfur, phosphate, sugar/polysaccharide, lipid/sterol, secondary metabolite, generic hydroxylases, oxidoreductases, esterases, and transferases, 'other' kinases, solute-binding proteins	14.28	**10.05**	↓	**10.42**	↓
Energy					
Respiration, glycolysis, TCA, electron transport, ATP synthesis	2.31	**4.47**	↑	**3.76**	↑
Photosynthesis					
Light reaction, dark reaction, chloroplast structure, chloroplast metabolism, chloroplast protein synthesis	9.09	**4.15**	↓↓	**6.52**	↓
Cell growth/development					
Cell division, organ/tissue/cell development, movement, senescence-related (unless documented as stress responsive)	1.81	**2.70**	↑	1.72	
Nucleic acid metabolism					
Transcription and RNA metabolism, DNA synthesis, DNA modification, chromatin modification	8.49	9.13		**5.30**	↓
Protein metabolism					
Protein synthesis , protein degradation, protein localization, protein modification, Generic protein-protein interacting polypeptides [including these domains: ankyrin repeats, tetra- and pentatricopeptide repeats (TPR, PPR), ARM, WWE], chaperones	9.13	**12.75**	↑	9.99	
Transport					
Ions, sugars, lipids, ABC-type, other small molecules, macromolecules, carrier proteins, proton translocators	3.75	**2.99**	↓	**2.30**	↓
Cellular trafficking					
Nuclear, organellar, cell wall/membrane proteins [including: plasma and tonoplast intrinsic membrane prots], cytoskeleton, ER/golgi, secreted proteins	3.34	2.88		2.76	
Signaling					
Hormone metabolism, receptors/receptor kinases/two component systems, protein kinases, protein phosphatases, transducers, second messenger metabolism, WD40, pleckstrin homology domain-containing proteins	6.24	6.89		**4.15**	↓
Defense/stress					
Disease, wounding, environmental extremes, salt/chemical/heavy metal tolerance	5.51	**8.76**	↑	**15.80**	↑↑
Unclassified					
Not enough information to assign to a specific Functional Category metal binding or zinc finger proteins with no catalytic/biological function information proteins containing leucine-rich repeats of unknown function hypothetical and expressed proteins of unknown function proteins of unknown function	19.92	21.60		17.28	
Unidentified					
No hits in BLAST analysis	16.14	13.44		20.00	

Wei et al. (2005). In contrast to the latter studies, we utilized photosynthesis as a distinct category and combined several aspects of protein biochemistry into a general category of protein metabolism. Because of the large number of ESTs that needed to be manually assigned, we did not utilize any of the subcategories previously used within each of the main categories. As in other studies, assignment of the annotations into one of the categories was based on a variety of search programs, including Expasy (Expert Protein Analysis System: http://

www.expasy.org/), InterPro (European Molecular Biology Laboratory-European Bioinformatics Institute: http://www.ebi.ac.uk/interpro/), and Entrez and PubMed (http://www.ncbi.nlm.nih.gov/).

The number of genes, represented as a percentage of the total number of genes in each of the three treatment categories, is presented in Table 4. Distinct differences, representing either an increase or decrease in the percentage of genes represented in a functional category, were observed in the two stress treatments relative to the control. Differences greater than 25% are highlighted in bold and identified with a single arrow, whereas greater than two-fold differences are noted in bold and identified by two arrows. Regarding the low-temperature treatment, after 24 h at 5°C there was a decrease in general metabolism and photosynthesis-related genes, and an increase in protein metabolism and defense/stress genes. In response to 2 weeks of a water deficit treatment, there was a decrease in general metabolism, photosynthesis, nucleic acid metabolism and a three-fold increase in defense/stress genes.

A comparison of the changes (increase or decrease) in the percentage of genes in each functional category in response to low temperature or water deficit is also presented in Fig. 1, where the percentage change is shown relative to the control. For example, in response to low temperature there was an increase of 3.62% in the number of genes in the functional category, 'protein metabolism' (Table 4). This represents an increase of 40% over the number of genes in this category relative to the control (Fig. 1). The same trends are present as indicated in the data in Table 3; however, the data are more readily visualized in Fig. 1.

Identification and quantification of sequences unique to each of the stress-related libraries

The upregulation of specific genes in the different tissue- and stress-specific libraries is presented in Table 5 and a similar analysis of downregulation is shown in Table 6. In order to be considered for inclusion in these analyses, any given gene needed to contain five or more sequences as part of a cluster. This was done to exclude genes whose absence may have been a reflection of random sampling error as previously discussed (Audic and Claverie 2006). The genes in Tables 5 and 6 are organized into the same functional categories previously presented.

Twenty-four hour low-temperature treatment resulted in a dramatic increase in defense-related genes in bark and xylem, ranging from around 15-fold to a potential

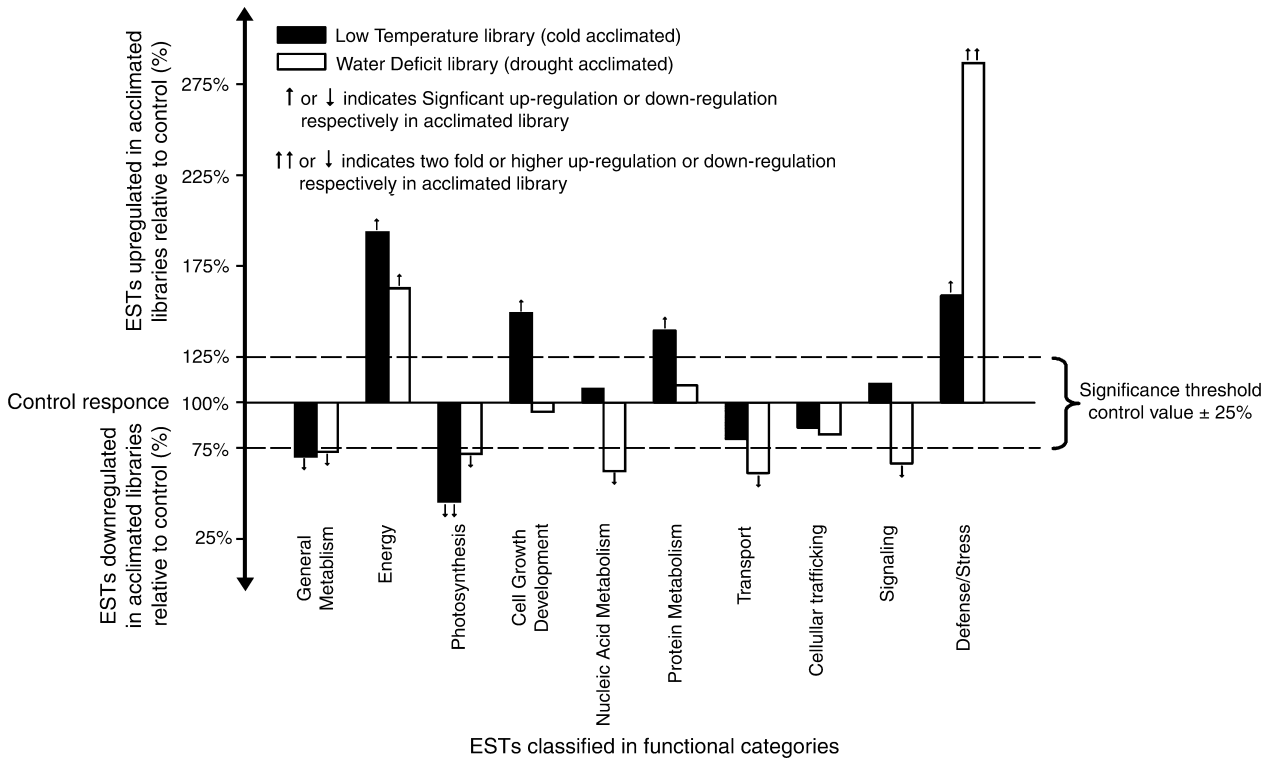

Fig. 1. A comparison of the increase or decrease in the percentage of ESTs in each of the functional categories in response to either low temperature or water deficit. Per cent changes are relative to the control which was set at 100%.

Table 5. Genes upregulated by stress treatment in different apple tissues. [a]Fold differences were computed by dividing the percentage of a given sequence in a treated tissue by the percentage of that sequence in the appropriate untreated tissue control. Percentages were calculated as the number of sequences in a contig containing five or more sequences, divided by the total number of cDNAs for that library × 100. If no equivalent sequence was found in the control library, the numbers four or one were used to calculate a range that could statistically represent an undetected sequence in the untreated tissue control. The sign, > indicates the maximum end of the range where a single sequence was assumed in the control library to prevent dividing by zero and indicates the fold increase of a given sequence over the control in response to a treatment. Differences potentially equal to or greater than 20-fold are highlighted in bold.

	Low temperature treatment fold increase over control[a]			Water deficit treatment fold increase over control[a]		
Annotations	Leaf	Bark	Xylem	Leaf	Bark	Root
Defense/stress						
Dehydrin Xero2		**31 to >122**				
Dehydrin Cor47	2.9 to >10	15			**12 to >48**	1.5 to >5.6
Peroxidase 42		17			**5.4 to >22**	1.8 to >6.8
cab-binding fam prot, ELIP	4.3	15		3.5		
Chalcone synthase	2.7	**28**				
Metallothionein-like prot		**47**	**15 to >61**		**16 to >64**	**5.8 to >22**
Thaumatin-like protein				2 to >8		
DNA J HSP			**5 to >20**			
17.6 kDa sm HSP				2 to >8	**5.4 to >22**	
18.1 kDa, class I HSP				**15 to >60**	**90 to >360**	
18 kDa HSP				**5.4 to >21**	**32 to >129**	
HSP 22 kDa ER					**6.5 to >26**	
25.3 kDa sm HSP				4 to >16	**6.5 to >26**	
HSP70					**5.4 to >22**	
HSP 81-1					**6.5 to >26**	
HSP 81-2						1.5 to >5.6
L-ascorbate peroxidase 1				3 to <12		2.4 to >9
Bet v I allergen fam prot (At1g70830)						1.5 to >5.6
Bet v I allergen fam prot (At1g24020)						5.6
Lipid transfer prot 3				2 to >8		
Ethylene-responsive transc coactivator					**6.5 to >26**	
Peptidyl-prolyl *cis-trans* isomerase	4 to >16	**7 to >28**		2 to >8	**5.4 to >22**	2.4 to >9
Energy						
Triosephosphate isomerase		4				
Fructose-bisphosphate aldolase	2.9 to >11		**7 to >28**	**6.6 to >26**		
General metabolism						
Starch synthase			**9 to >36**			
Caffeoyl-CoA 3-O-methyltransferase			**8 to >32**			
Mannose 6-Phos reductase (NADPH dep)	5			2.7		
Glycerophosphoryl PO diesterase fam	3.6 to >15					
Beta amylase	2.9 to >11					
Phosphoglycerate kinase				2 to >8		
Carbonic anhydrase 1				3.3 to >13		
Dienelactone hydrolase fam prot						3
Acid phosphatase						5.6
(S)-2-hydroxy-acid oxidase (peroxisomal)				2.9 to >11		
Photosynthesis						
cab-binding prot LHCII, type 1	14	**5.9 to >25**		3.3 to >13		
cab-binding prot LHCII, type III				2 to >8		
cab-binding prot, LHCA2				3.3 to >13		
cab-binding prot, LHCB4.2				2 to >8		
cab-binding prot, LHCB4.3				2 to >8		
cab-binding prot, LHCB6	2 to>8			2 to >8		
photosys II 22 kDa chloro prot, CP22	4 to >16			2.9 to >11		
PSII, 10 kDa prot				3.8		

Table 5. Continued

Annotations	Low temperature treatment fold increase over control[a]			Water deficit treatment fold increase over control[a]		
	Leaf	Bark	Xylem	Leaf	Bark	Root
Ferredoxin, PETF	2.5			**5.2 to >21**		
cab-binding prot, type III				**6.2 to >25**		
cab-binding prot 2				1.6		
cab-binding prot 4				2.9 to >11		
photo sys II oxy-evolving complex 23				2.5 to >10		
Oxygen-evolving prot				1.4		
photo sys I rxn cent subunit IV				4 to >12		
Plastocyanin				3.3 to >13		
Rubisco SSU 1A	**7.9 to >31**					
Rubisco SSU 3B				**8.6 to >34**		
Rubisco activase	2 to >8			**9.3 to >37**		
CP12 domain-contain prot				2 to >8		
Transport						
mito import subunit Tim17		**5 to >20**				
Signaling						
Calmolulin-7 (CAM7)		**5 to >20**				
Protein metabolism						
eIF SUI1		**5 to >20**				
eIF 5A		**9 to >34**				
40S ribo prot, S4		**9 to >34**				
60S ribo prot L19	2 to >8					
Glutamine synthetase	>8.5					
Cysteine synthase	2 to >8					
Ubiquitin-conjug enzyme 10		5 to >20				
Polyubiquitin (UBQ4)		**6 to >24**				
Polyubiquitin (UBQ10), senesc assoc prot	3.2 to >13					
Cell growth/development						
Dormancy-assoc (DRM1)		**8 to >32**	**9 to >36**			
Nucleic acid metabolism						
gly-rich RNA-binding prot (GRP7)		**15 to >60**				
RNA binding			**10 to >40**			
Histone H1.2		**6 to >24**				
Histone H3	3.2 to' >13	**11 to >44**	**9 to >36**	2.5 to >10	**10.8 to >43**	2 to >8
High mobility group, B3		4				
Cell/intracell trafficking						
Tubulin alpha-6 chain		**5 to >20**				
Plasma intrinsic protein			**11 to >44**			
ADP-ribosylation factor	2.9 to >11		**9 to >34**			
Unclassified						
Protease inhib/LTP			**5 to >20**			
Protease inhib/LTP			**8 to >32**			
Metal ion binding			**5 to >20**			
Translationally controlled tumor fam prot		**5.4 to >22**			**8.6 to >35**	

high over 100-fold (dehydrin similar to Xero2). Interestingly, there were only comparatively modest increases in these genes in leaves. Substantial increases in genes associated with nucleic acid metabolism were also noted in bark and xylem tissues, whereas leaves showed more significant increases in genes involved in general metabolism, photosynthesis and protein metabolism.

In contrast, water-deficit-treated leaves showed substantial (>5-fold) increases in a wide variety of photosynthetic genes and in many of the stress-related genes. The largest variety of genes and the greatest differences were observed with the defense/stress genes from water-limited bark tissues. These ranged from 22-fold (peroxidase and HSP70) to over 300-fold (HSP18.1). The

Table 6. Genes downregulated by stress treatment in different apple tissues. LRR, leucine-rich repeats [a]Fold differences were computed by dividing the percentage of a given sequence in the appropriate control by the percentage of that sequence in the appropriate treated tissue. Percentages were calculated as the number of a given sequence (at least five or greater) in a control library divided by the total number of cDNAs in that library × 100. If no equivalent sequence was found in the treated tissue library, the numbers four and one were used to calculate a range that could statistically represent an undetected sequence in the treated tissue. The sign, >, indicates the maximum end of the range where a single sequence was assumed for the treated library to avoid dividing by zero, and reflects the fold-increase in the control relative to the treated tissue. Differences potentially equal to or greater than 20-fold are highlighted in bold.

	Low temperature treatment fold increase over treated[a]			Water deficit treatment fold increase over treated[a]		
Annotations	Leaf	Bark	Xylem	Leaf	Bark	Root
Defense/stress						
Similar to ABA-inducible protein	1.1 to >4.2			1.1 to >4.2		
Superoxide dismutase	0.8 to >3.0			0.8 to >3.0		
Heavy-metal domain prot		0.4 to >1.3	0.4 to >1.3		0.4 to >1.3	1.3 to >4.7
Chalcone synthase						1.1 to >4
Glutathione S-transferase		0.3 to >1.2	0.3 to >1.2		0.3 to >1.2	
General metabolism						
Glycine dehydrogenase	1.1 to >4.2			1.1 to >4.2		
Methylenetetrahydrofolate reductase 2	3 to >12			3 to >12		
Adenosylhomocysteinase	2.6 to >10			2.6 to >10		
Geranylgeranyl reductase	**7.8 to >30**			**7.8 to >30**		
Prephenate dehydratase-like (production of Phe)	1 to >3.5			1 to >3.5		
Sorbitol dehydrogenase	1 to >3.5			1 to >3.5		1.3 to >4.7
Cytochrome P450						3 to >11
Hydroxymethylglutaryl-CoA synthase						2 to >7.2
Lactoylglutathione lyase/glyoxalase						1.1 to >4
Alkaline alpha galactosidase						3.5 to >13
Lectin-like protein		0.4 to >1.3	0.4 to >1.3		0.4 to >1.3	
UTP-glucose-1-phosphate uridylyltransferase		0.3 to >1.2	0.3 to >1.2		0.3 to >1.2	
Adenylate/nucleotide kinase family		0.3 to >1.2	0.3 to >1.2		0.3 to >1.2	
Energy						
Glyceraldehyde 3-phosphate dehydrogenase, cytoplasmic						1.5 to >5.5
Fructose-bisphosphate aldolase						2 to >7.2
Photosynthesis						
Chl *a*/*b*-binding protein (LHCB2.2)	**48 to >183**			**48 to >183**		
Chloroplast nucleotide DNA-binding protein, CND41-like	2 to >7.7			2 to >7.7		
PSII reaction center W (PsbW-like)	3.5 to >14			3.5 to >14		
cab-binding prot 2	0.8 to >3.0			0.8 to >3.0		
Glyceraldehyde 3-phosphate dehydrogenase, chl	0.8 to >3.0			0.8 to >3.0		
Chl *a*/*b*-binding protein CP26	**35 to >133**			**28**		
rubisco subunit binding-protein, alpha subunit	1.5 to >5.8					
Plastocyanin-like domain-containing prot						1.1 to >4
Blue copper protein		0.3 to >1.2	0.3 to >1.2		0.3 to >1.2	
Transport						
Sorbitol/mannitol transporter	1.1 to >4.2			1.1 to >4.2		
Mitochondrial substrate carrier family protein	0.8 to >3.0			0.8 to >3.0		
Signaling						
Casein kinase II alpha chain 1	0.8 to >3.0			0.8 to >3.0		
LRR protein kinase		0.4 to >1.3	0.4 to >1.3		0.4 to >1.3	
Protein kinase		0.3 to >1.2	0.3 to >1.2		0.3 to >1.2	
Protein metabolism						
40S ribosomal protein S2	2.7 to >11			2.7 to >11		
40S ribosomal protein S6	2.7 to >11			2.7 to >11		
40S ribosomal protein S10		0.3 to >1.2	0.3 to >1.2		0.3 to >1.2	
40S ribosomal protein S16	1.4 to >5.3			1.4 to >5.3		
40S ribosomal protein S27	1.8 to >7			1.8 to >7		

Table 6. Continued

Annotations	Low temperature treatment fold increase over treated[a]			Water deficit treatment fold increase over treated[a]		
	Leaf	Bark	Xylem	Leaf	Bark	Root
60S ribosomal protein L6	1 to >3.5			1 to >3.5		
26S proteasome alpha subunit		0.4 to >1.3	0.4 to >1.3		0.4 to >1.3	
Cell growth/development						
Ripening regulated prot		0.3 to >1.2	0.3 to >1.2		0.3 to >1.2	
Nucleic acid metabolism						
PUR alpha-1, single-stranded DNA-binding protein	1.5 to >5.8			1.5 to >5.8		
KH domain-like protein (RNA binding domain)	0.8 to >3.0			0.8 to >3.0		
histone H2B	1.2 to >4.7			1.2 to >4.7		
Rap2.2 transcription factor						1.1 to >4
Zinc finger (AN1-like) DNA binding protein		0.3 to >1.2	0.3 to >1.2		0.3 to >1.2	
Cell/intracell trafficking						
Tubulin beta-8 chain (TUB8)	1.1 to >4.2			1.1 to >4.2		
Vacuolar ATP synthase	2.4 to >9.3			2.4 to >9.3		
Plasma instrinsic proteins						5.2
ER retrieval protein (Rer1B)		0.3 to >1.2	0.3 to >1.2		0.3 to >1.2	

increase in defense genes in roots was considerably more modest than that observed in bark, although the defense gene category was the most widely represented of all the functional categories in roots.

With a few exceptions, the overall differences in downregulated genes were nominal compared with differences in upregulated genes. For example, in low-temperature-exposed leaves, there were decreases in several photosynthetic genes (which did not overlap the photosynthetic genes observed to increase after treatment), but only a few were more than 100-fold reduced (LHCB2.2 and CP26, both Chl *a*/*b*-binding proteins). Similar differences were also noted in the water-limited leaves. Although some metabolic genes (general and protein) were repressed 10-fold or more in both the low temperature and water deficit leaves, most of the genes were reduced less than 10-fold in all treatments and tissues.

To verify the quantitative EST results, semiquantitative RT-PCR was performed using primers for select genes representing up- or downregulated sequences. These reactions were run using cDNA templates representing RNAs from each treatment and tissue. In general, the RT-PCR analyses confirmed the direction and quantification of the EST analysis and supported the tissue specificity observed in the EST libraries (Figs 2 and 3).

Discussion

Our analysis of apple ESTs from six treatment/tissue libraries and three controls was in general agreement with other studies examining global gene expression in response to low temperature and water deficit. More than two-thirds of the sequences in our libraries could be identified based on similarity to sequences of known function, and that fraction increased to three-fourths if sequences of unknown function were included. In most cases, less than 20% of the sequences analyzed showed no similarity to sequences in the databanks. Reliability of the sequence matches was enhanced by the use of relatively large insert sizes for the ESTs, thus increasing the lengths of potential match/mismatch.

Comparison of the library collections by sequentially subtracting common sequences led to the identification of ESTs unique to a particular tissue/treatment combination. For five of the libraries, the ratio of unique to similar sequences was around 0.5 (Table 2); interestingly, this ratio in the low-temperature-treated xylem library (MdLTX) was closer to 0.75. In addition, the ratio decreased with each subtraction for all the libraries except the MdLTX library, suggesting that this library had considerably fewer sequences in common with the other libraries. As subtraction of the stem control ESTs did not result in the removal of many sequences, the larger ratio of unique-to-similar sequence may reflect the uniqueness of the xylem's response to low temperature. We also observed a significant increase in the number of genes unique to cold stress in the leaf libraries. This may imply that apple leaves respond in a manner similar to herbaceous tissue, whereas the response of bark and xylem tissues is more specific to woody plants. Benedict et al. (2006) observed that CBF genes (a major cold regulon) in *Populus* responded very differently to cold treatment in annual vs perennial tissues and induced

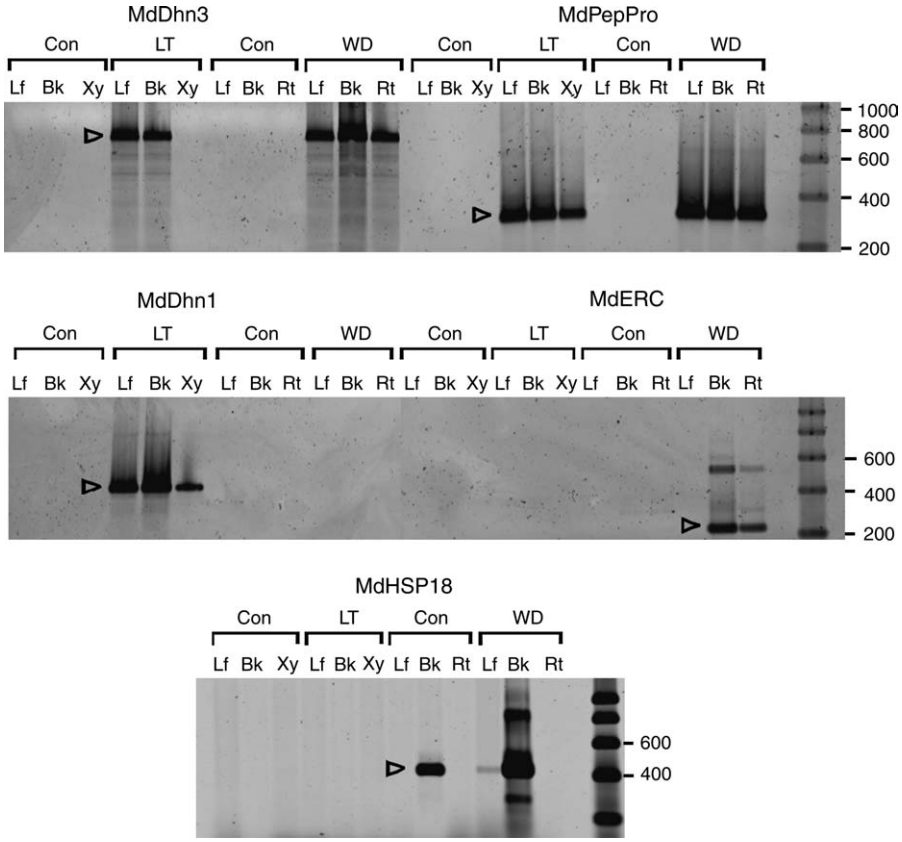

Fig. 2. RT-PCR confirmations of EST quantification of upregulated LT- and WD-responsive genes. RNA was extracted from each tissue and treatment as specified in Materials and methods. cDNAs were synthesized and normalized so that equal cDNA masses served as templates in the PCR reactions. PCR products were electrophoresed and separated in 1% agarose gels. DNAs were visualized with SYBR Gold. Stained gels were scanned in an image analyzer and select bands were quantified using ImageQuant software. Reactions for five different upregulated genes are presented. Molecular mass markers are indicated on the right. Arrowheads indicate the product size predicted from sequence information. Genes indicated are listed in footnote 'b' of Table 1. Treatments and tissues are indicated as follows: Con: untreated control; LT, trees exposed to 5 °C for 24 h; WD, water was withheld from trees to 45% saturation for 2 weeks; Lf, mature leaf; Bk, bark; Xy, xylem; Rt, root.

different sets of genes. These authors suggested that these differences may reflect the evolution of different roles for these 'master switches' in annual and perennial tissues of woody species. In a study on Eucalyptus, Kayal et al. (2006) noted a differential response to cold by different CBF genes. *EguCBF1a* was transiently expressed in response to a severe 'cold shock' treatment, while *EguCBF1b* responded more readily and over an extended period of time to a 'milder' cold treatment. These observations also highlight differences between herbaceous and woody perennial plants.

In transcript profiling studies, broad categories of gene function are assigned to facilitate linkage between gene identities and biological/biochemical roles. The creation of categories summarizing biological functions is as varied as the number of studies reported, and basic categories are often joined or subcategorized to reflect some constraint or pertinence to the reported results. We used the functional categories reported in Dhanaraj et al. (2004) and Wei et al. (2005) with a few modifications. We combined the categories of 'protein destination' and storage with 'protein synthesis' into a category termed 'protein metabolism', which also includes protein degradation and modification. We also created a separate 'photosynthesis' category distinct from the 'energy' category. In addition, we combined Intracellular Trafficking with Cell structure because the two groups are closely linked. These categories are intended to convey general information about specific classes of genes and are not expected to precisely represent a function or role for any specific gene. This is partly because many proteins have multiple roles, some of which fall into different categories and because many gene families contain individual members that are linked to different roles.

Estimating the number of ESTs that could be classified into our functional categories resulted in several general

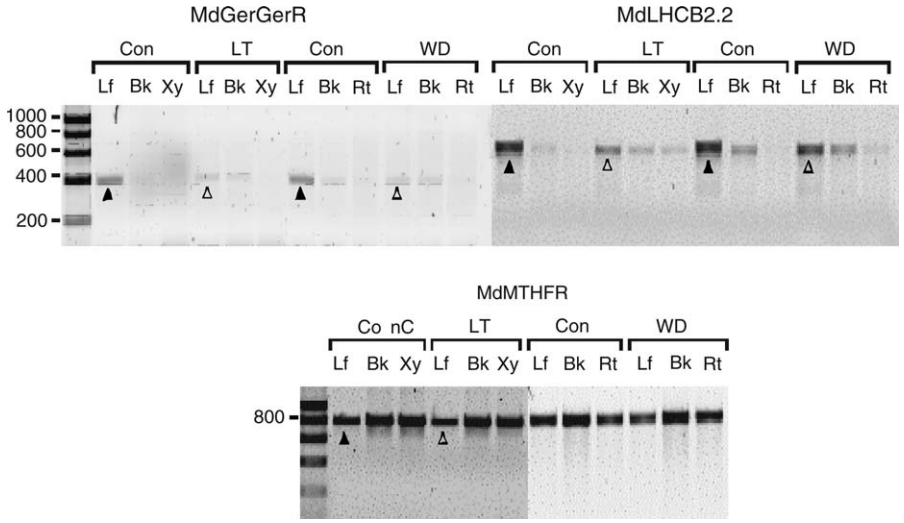

Fig. 3. RT-PCR confirmations of EST quantification of downregulated LT- and WD-responsive genes. Reactions and products were analyzed as described in the caption to Fig, 2. Reactions for three different downregulated genes are presented. Molecular mass markers are indicated on the left. Genes indicated are listed in footnote 'b' of Table 1. Arrowheads reflect the abundance of the indicated PCR product bands, as determined by densitometry: ▲, more abundant; △, less abundant. Treatments and tissues are indicated as follows: Con, untreated control; LT, trees exposed to 5°C for 24 h; WD, water was withheld from trees to 45% saturation for 2 weeks; Lf, mature leaf; Bk, bark; Xy, xylem; Rt, root.

trends. With a few exceptions, regulation of sequences responding to either LT or WD showed similar tendencies, i.e. if sequences in a given category were upregulated by LT, they were also upregulated by WD. The three exceptions were Cell Growth/Development, Nucleic Acid Metabolism, and Signaling where sequences were underrepresented in WD libraries but overrepresented in LT libraries. This observation may reflect the difference in exposure time (24 h for LT vs 2 weeks for WD) between the two treatments, as signaling pathway components, growth/developmental regulators and transcription factors usually respond rapidly to stimuli and may be rapidly deactivated. In terms of the LT treatment, more photosynthetic genes were suppressed and more energy-related genes upregulated than seen in WD tissues. In contrast, water deficit treatment resulted in a substantial increase in defense-related genes compared with the LT libraries. Again, this may relate to differences in the length of time the stress was applied to the tissues.

Unigenes derived from contigs containing at least five independent ESTs were quantified in the libraries and used to compute differences between control and treated tissues. To verify these results, semiquantitative PCR was used to assess the abundance of select genes representing up and down regulation in freshly synthesized cDNAs. These results were remarkably consistent with estimates from the EST determinations in terms of both treatment and tissue specificity (cf. Tables 5 and 6 with Figs 2 and 3). In those few cases where there was a difference, e.g. a PCR product representing peptidylprolyl *cis-trans* isomerase seen in LT-treated xylem but not in the ESTs, the discrepancy could be attributed to the statistical threshold limit of five ESTs: there were only three ESTs representing peptidylprolyl *cis-trans* isomerase in the LT xylem library. Downregulated genes were somewhat problematic in that most were members of multigene families with a high degree of sequence conservation. The small size of the ESTs made primer design for a specific family member difficult. As a result, in some cases the downregulated genes could have been masked by closely related family members that did not show a similar change. A possible example is seen in Fig. 3 for the methylenetetrahydrofolate reductase gene which in the EST libraries was downregulated in leaves of both stress treatments; in the PCR gels, only the LT-treated leaves show any detectable decrease.

Quantification of ESTs revealed a modest increase in a number of photosynthetic genes in the leaves of low-temperature- and water-deficit-treated apple (Table 5), although a greater variety of these genes responded positively to dehydration. These genes primarily represented PSII proteins, including several Chl *a*/*b*-binding family members. During abiotic stress exposure (e.g. cold acclimation), excess light energy in the form of electrons accumulates in Q_A (plastiquinone A; Öquist and Huner, 2003), and this reduced capacity to transfer electrons through PSII requires adjustments in gene expression for long-term adaptation. This could account for the observed increase in PSII gene abundance in our stressed apple leaves. Furthermore, in water-stressed apple leaves,

there was an increase in PsbP (oxygen-evolving complex, subunit 23) by as much as 10-fold. A recent study has demonstrated that PsbP is essential for the regulation and stability of PSII (Ifuku et al. 2005); this observation is compatible with long-term adaptation of PSII components in the water-deficit-treated apple leaves.

In addition to proteins associated with the light reactions of photosynthesis, we also observed increases in rubisco small subunit (SSU3B) and rubisco activase in both stress-treated leaves. This result is consistent with limited CO_2 availability and its repressive affect on electron flow associated with abiotic stresses (Asada 1999, Hirotsu et al. 2005). In this case, the observed increases in transcripts for rubisco small subunit and in its activation protein could be a compensatory mechanism for modulating the adverse effects of the stress treatment. In the case of cold-treated plants, the duration of cold exposure may have been short enough not to have severely damaged the photosynthetic apparatus, but long enough to have reduced some of the photosystem components (e.g. LHCB2.2). Upregulation of some PSII genes in low temperature may have been required to compensate for this reduction. In the case of the water-deficit-treated leaves, although the stress was over a longer period, it was gradual and may have been less damaging to the photosynthetic apparatus. In support of this, sampled leaves did not show any typical symptoms of photosynthetic stress, such as yellowing or streaking, nor did they show signs of extreme dehydration (wilting or curling). Because of the extensive upregulation of photosynthetic genes in water-deficit-treated leaves, it is possible that these leaves might still be capable of photosynthesis, although likely at efficiencies below optimal.

A striking feature of the water deficit upregulated genes in apple was the substantial increase in heat shock proteins seen primarily in leaves and bark, mainly involving the small molecular weight class. Heat shock genes are generally responsive to multiple abiotic stresses, as this class of proteins is known to aid in maintaining cellular proteins in their functional conformations during stress (Wang et al. 2004). Early studies in *Arabidopsis* subjected to a rapid, severe dehydration treatment identified upregulated heat shock proteins (HSP) that were members of the 70 and 90 kDa classes (Kiyosue et al. 1994). Similarly, Rizhsky et al. (2004) observed mainly the larger heat shock protein class (HSP70 and HSP90 families) represented in sequences upregulated by drought stress in *Arabidopsis*, in contrast to both small and large heat shock families that were upregulated by heat shock alone. In pine needles during water deficit stress, a low molecular weight heat shock protein was one of six upregulated polypeptides identified from 2-D gels (Costa et al. 1998). In support of a protective role for small HSPs during abiotic stress, overexpression of HSP17.6A in *Arabidopsis* conferred both salt and drought tolerance (Sun et al. 2001).

In *Arabidopsis* regulatory proteins that respond to cold or drought treatment bind to a core *cis*-element in the promoters of a subset of genes associated with stress response. The DREB1/CBF proteins are thought to activate expression of genes that respond primarily to cold (Gilmour et al. 1998, Liu et al. 1998), whereas DREB2 and CBF4 proteins are believed to be associated with drought-responsive gene expression (Haake et al. 2002, Liu et al. 1998). Deletion of a negative regulatory domain in DREB2A results in constitutive activation of the transcription factor and the concomitant expression of downstream drought- and salt-responsive genes (Sakuma et al. 2006a). Recent analysis of the genes up-regulated in *Arabidopsis* plants transformed with the deletion-activated DREB2A under a 35S promoter identified two heat shock factors, members of both high and low molecular weight HSP families (Sakuma et al. 2006b). Although the response of the HSPs *in vivo* to drought stress was weak, both possessed DREB core elements in their promoters, suggesting that these elements were functional, but might not reach maximum expression levels under the conditions/tissues specified in the study. Campalans et al. (2001) also reported an elevated low molecular weight HSP in drought-stressed almond plantlets. These results support our observation of the up-regulation of HSPs in leaves, bark and roots (HSP81-2) of water-deficit treated plants. In our study, a DREB1 homolog EST was identified in the LT-treated xylem library derived from trees exposed to 5°C for 24 h (Supplementary material Table S1). Absence of a DREB1 EST homolog in the other LT-treated tissues may simply reflect its low copy number and the number of samples sequenced from each library.

Downregulation of a subset of ribosomal protein genes was observed in both the cold treated and water stressed leaves of apple. A recent study of translation regulation in leaves of moderately dehydration-stressed plants of *Arabidopsis* indicated that a large portion of mRNAs were no longer associated with polysomes, while over half of dehydration-inducible mRNAs (induced 2-fold or greater) were still associated with polysomes during the water stress treatment (Kawaguchi et al. 2004). Although some of the translationally repressed mRNAs were reduced in abundance in stressed leaves, others, including 73 cytosolic ribosomal protein mRNAs, were not appreciably altered in abundance. However, these mRNAs were significantly less likely to be associated with polysomes in stressed leaves compared to controls, suggesting that initiation of translation of a relatively large fraction of cellular mRNAs is suppressed during water deficit treatment. In contrast to the *Arabidopsis* study, the

EST data presented here suggest that reduced ribosomal protein mRNA abundance may lead to suppression of translation in cold or water-deficit treated leaves of apple. It has been well documented that general suppression of translation selectively favors mRNAs with better translation initiation efficiency and could therefore contribute to the differential expression of specific subsets of genes, e.g. stress-responsive mRNAs.

The overall effect of low-temperature and water-deficit stress on gene expression in apple is similar to that reported in other global genomic studies (Bassett et al. 2006, Dhanaraj et al. 2004, Dubos and Plomion 2003, Kayal et al. 2006, Nanjo et al. 2004, Street et al. 2006, Wei et al. 2005). For example, dehydrins are observed to be significantly upregulated in most LT and WD experiments, and different dehydrin family members are usually observed to respond to cold (e.g. *PpDhn1* and *3*) or water deficit (e.g. *PpDhn2*). In addition, several classes of pathogen-related genes have been reported to increase in response to low temperature or water limitation, including peroxidases, Bet V I allergens, and thaumatin-like proteins. However, in contrast to several reports (Dhanaraj et al. 2007, Fowler and Thomashow 2002, Wei et al. 2005, Wong et al. 2006), we did not observe an increase in ELIPs in response to either LT or WD in apple, neither did we observe expression of this gene family in peach bark in response to LT (Bassett et al. 2006). As ELIPs are associated with protection of PSII (Montane and Kloppstech 2000) against light-induced stress, it is surprising that they are not represented in LT or WD responsive tissues. It is possible that peach and apple have evolved a different adaptation mechanism to stresses that produce excess photons, e.g. upregulation of other PSII components (see above).

Our study also provides new information on tissue-specific responses to LT and WD. It is clear from Tables 5 and 6 that bark and leaves respond differently to both stresses, although there is some overlap in the identity of genes expressed, particularly those associated with defense. Where there is overlap, the differences between tissues appear to be quantitative (cf. 18 HSP class between bark and leaf in response to WD) by both EST analysis and RT-PCR.

In this study, we examined short-term (24 h) gene expression in apple trees exposed to low temperatures, and we also measured changes in expression in trees subjected to a gradual, but long-term (2 weeks) drought period. Different tissues were sampled to fully profile expression in these trees. Our results are similar to other studies in woody and herbaceous plants in that similar groups of genes are found to be up- and downregulated in all studies of low temperature and water deficit responses so far reported. However, we observed some unique aspects, including the upregulation of a subset of low molecular weight (approximately 18 kDa) heat shock proteins in leaf and bark in response to water deficit. We have also provided information on the response of specific tissues (leaf, bark, xylem and root) to both low temperature and water deficit. This information may be utilized to understand how these specific tissues respond and adapt to environmental stress.

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Supplementary material

The following supplementary material is available for this article:

Table S1. List of annotated ESTs obtained from the different cDNA libraries used in this study. See Materials and methods for description of the various cDNA libraries.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1399-3054.2008.01063.x

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